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Dengue virus non-structural protein 1 binding to thrombin as a dengue severity marker: Comprehensive patient analysis in south Taiwan

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ABSTRACT

Background: Previously we identified a complex of non-structural protein (NS) 1 – Thrombin (NST) in dengue infected patients. Here, we investigated how the concentration of NS1 and NST differ in various dengue severity levels as well as their demographic and clinical features. Several comorbid (hypertension, diabetes, and chronic renal failure) often found in dengue patients were also measured and analyzed.

Methods: A total of 86 dengue patients (52 not severe and 34 severe), were enrolled and had their blood taken. Blood samples were further verified for clinical blood parameters, including liver and renal function tests and serologic assays (NS1 and NST). Patients' severity was grouped based on WHO 2009 classification, which separates patients into dengue without warning signs (DNWS), dengue with warning signs (DWWS), and severe dengue (SD). DWWS is explained as DNWS with warning signs (persistent abdominal pain, persistent vomiting, liver enlargement, bleeding (any kind), fatigue, and restlessness). SD are those with severe plasma leakage, severe bleeding, or severe organ impairment. Multivariate regression analysis was used to predict the role of NST on the dengue severity development and receiver operating characteristic (AUROC) test was utilized to evaluate separability.

Results: The analysis revealed that NS1 significantly impacts the disease outcome (p 0.018, OR = 2.467 (1.171–5.197)) but not beyond the effect through NST (p 0.108, OR = 0.085 (0.004–1.719)). We also prove that NST was a better severity biomarker compared to NS1, as it can predict progression from DNWS to DWWS (AUC: NS1 = 0.771**, NST = 0.81**) and SD (AUC: NS1 = 0.607, NST = 0.754*) significantly.

Conclusions: This finding suggests the importance of NST in mediating the NS1 effect to promote dengue severity progression and its promising capability as an acute stage dengue severity biomarker.

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1. Introduction

Dengue is a significant public health issue caused by dengue virus (DENV) infection. In 2023 only, five WHO regions reported more than five million cases and more than 5000 dengue related deaths, almost reaching a history all-time high.[1] Dengue severity was classified by WHO in 2009 and is grouped into dengue without warning signs (DNWS), dengue with warning signs (DWWS), and severe dengue (SD).[2] DENV comes from the *Flaviviridae* family and is grouped as an RNA virus. Its genome encodes three structural proteins and seven nonstructural (NS) proteins, with a total genome length of 11 kb.[3,4] The virus, host, and environmental factors are just a few of the variables that affect how severe the clinical course is.[5] Various reports showed the importance of appropriate dengue monoclonal antibodies (mAbs) concentration to neutralise the infection effectively. This dose-related effectivity is commonly required for prM-specific mAbs followed by some E-specific mAbs.[6] In 2010, Schieffelin et al. isolated three human E-protein specific mAbs, and two showed no neutralising but enhancing activity.[7] The more recent report presented prM-specific mAbs, a major component of human-specific response against DENV. Although showing high inter-serotype cross-reactivity, even in high concentrations, they do not neutralise infection but might cause ADE or Antibody Dependent Enhancement, which promotes intracellular viral replication.[8] The downside of high viral titers are known to lead to cytokine storms or cytokine release syndrome (CRS), an excessive release of proinflammatory cytokines from the body, that may further promote disease progression in the patients.[9,10]

Regardless of its complicated pathogenesis, DENV infection has several severity manifestations that might be difficult to predict in its acute phase. The similarity of the symptoms is the main reason it is so difficult to predict its severity progression, as the majority of the patients only experienced DNWS, the mildest form of dengue disease. The diagnosis of DNWS is made based on the WHO 2009, referring to when acute high fever is accompanied by the minimum of these symptoms: pain behind the eyes, severe headache, nausea, muscle and joint aches, vomiting, rash, and swollen glands. Some patients might develop warning signs, including persistent vomiting, persistent abdominal pain, bleeding (any kind), liver enlargement, fatigue, and restlessness, and are thus diagnosed with DWWS. Severity progression from both DNWS and DWWS to SD is quite rare. Only five percent of these patients will develop SD, accompanied by severe plasma leakage, bleeding, and organ impairment. However, the survival rate of SD is highly influenced by access to medical care and the early detection of the case.[2] It is still a problem nowadays since the widely available dengue severity prediction mostly relies on the warning signs and its clinical symptoms, which might develop too late.

One of the most well-known viral proteins, non-structural (NS)-1, is produced inside the human host or the mosquito cells infected by DENV.[3] In clinical settings, NS1 is commonly used as a dengue infection marker on the first three days of infection, as the NS1 concentration peaks around the first day post fever.[11] Dengue NS1 is known as a virus replication cofactor and is assembled inside infected cells. Meanwhile, outside the cells, NS1 promotes the degradation of complement protein C4.[12–14] In addition, NS1 is also known to directly trigger an increase in endothelial permeability or induce the release of cytokines from peripheral mononuclear immune cells.[15,16] Due to this discovery, numerous studies have linked the NS1 titers with the severity of dengue pathogenesis and proposed NS1 as a dengue severity biomarker. Several of these studies have shown that NS1 does have promising capabilities as a dengue severity marker, especially when the NS1 rapid test results are positive over day five of the infection.[17,18] However, this severity prediction method is still limited in many aspects, and therefore, the search for a new severity marker is still ongoing. In 2012, we previously reported the findings of NS1 binding to thrombin/prothrombin (NST) and the potency of prothrombin inactivation.[19] Thrombin and prothrombin have an active role in blood coagulation, which is commonly disrupted in dengue infection. This complex might be majorly involved in the vulnerability of bleeding in dengue patients, especially in severe conditions where heavy bleeding is one of the symptoms. In this paper, we investigated the relationship between NST and dengue severity and evaluated its potential as a dengue severity marker. We also tried to identify clinical and demographic circumstances that might influence its concentrations.

2. Materials and methods

2.1. Ethics statement

Before participating in the study, each subject consented to be included. The study was carried out in compliance with the Declaration of Helsinki principles. Protocols were approved and followed the Institutional Review Boards of Taipei Medical University (reference number: N201801042) and National Cheng Kung University Hospital (reference number: A-BR-101-140), which were established and operated following the rules and regulations of Good Clinical Practice.

2.2. Selection of participants

Acute phase dengue patients were recruited at National Cheng Kung University Hospital from 2015 to 2016 (n = 86). All included patients were proven positive for dengue by rapid test for NS1 antigen or dengue antibody detection. Patient demographic information and clinical conditions were recorded and used in the analysis.

2.3. Definition of variables

A standardized data collection form was used to gather demographic information, hospital stay, clinical signs and symptoms, laboratory examination, and dengue severity classification. Patients younger than 18 years old were excluded. Age was grouped as adults (18–65 years old) and seniors ($\geq$65 years old).[20] Sex was classified as male or female based on the sex assigned at birth. Infection status was defined from serology examination results. Dengue patients were categorized based on IgG positivity and the IgM/IgG ratio. Patients with secondary infection exhibited positive IgG test or an IgM/IgG ratio $<$1.2, while those with primary infection had negative IgG or had an IgM/IgG ratio $\geq$1.2.[21–23] The day of fever was measured starting from the onset of the patient's fever symptoms. Dengue patients with comorbid conditions were grouped according to their comorbidity (88 % of the cohort had either diabetes mellitus (DM), chronic renal failure (CRF), or hypertension (HT)). Dengue patients' febrile phase starts from the onset of fever (Day zero) to Day three, where the critical stage further develops around Days four to seven post-fever onset.[24]

2.4. Definition of dengue severity

We classified dengue severity using the WHO 2009 criteria for DENV infection, which was determined by the existence of dengue warning signs. Mild disease or DNWS (group A) accounted for patients diagnosed with DENV infection who did not develop warning signs. Group B is those who were diagnosed with dengue and were developing warning signs, including persistent abdominal pain, persistent vomiting, liver enlargement, bleeding (any kind), fatigue, and restlessness (DWWS or might also be referred to as moderate). The severe dengue group (SD or Group C) included those who showed signs of severe plasma leakage, severe bleeding, or severe organ impairment.[24] Any dengue severity treated in intensive care units would also be included in group C.

2.5. Clinical blood parameters, liver and renal function tests, and dengue confirmation tests

The Department of Pathology at National Cheng Kung University

Hospital used standardized protocols to test the clinical blood parameters of the patients. The standard complete blood count test was used to determine the total number of circulating white blood cells (WBCs), hemoglobin (Hb), hematocrit (Hct), and platelet counts. In addition, glutamic pyruvic transaminase (GPT) and creatinine (Cr) were obtained from definitive liver and renal function tests. Additionally, confirmation of DENV diagnosis was performed using one or more examinations: positive for plasma NS1 antigen, dengue IgM antibodies detected using a kit (Bioline Dengue Duo™; Standard Diagnostics, Seoul, Korea), or DENV RNA detected using real-time reverse transcriptase-polymerase chain reaction (RT−PCR) (TIB Molbiol, Lightmix kit; Roche Applied Science, Berlin, Germany).[25]

2.6. ELISA detection of NS1 and NST concentrations

For viral NS1 measurement, in-house developed NS1 ELISA with anti-NS1 antibody were used (R&D Systems).[26] NST levels were measured according to previous work.[19]

2.7. Data analysis

Missing values in some variables (Hb, Hct, Cr, and GPT; Supp Table 1) were replaced with each group's median values. Dengue patient continuous variables (age, day of fever, hospital stay, comorbid number, WBC, Hb, Hct, platelet, GPT, Cr, NS1, and NST) were evaluated using the independent-samples Kruskal−Wallis test. The categorical variables (sex, infection, DM, hypertension, and CRF) were tested using the Wilcoxon test. Multivariate regression analysis was conducted to identify if either NS1 or NST were correlated with the outcome after adjustment for their confounders. Principal component analysis (PCA) was conducted to reduce the number of covariates as the allowance of sample size available. We determined a variable as a confounder if: (1) it was correlated to either NS1 or NST in addition to its correlation to the outcome; and (2) the hypothetical direction of the correlation was from the variable to NS1/NST/outcome. The hypothetical direction of the variables correlation were determined using known knowledge. If a variable had missing values, it was only included in this analysis if it was MCAR (missing completely at random). We imputed such variables using multiple imputations by chained equation (MICE). The missing values were imputed 10 times by the predictive mean matching method. Statistical tests showed no difference before and after imputation for the imputed variables. All tests were conducted with a 95 % confidence interval (95 % CI). Statistical significance was set at $p < 0.05$. These analyses were performed using IBM SPSS Statistics (version 27) and GraphPad Prism (version 9.5.0), and graphs were generated from GraphPad Prism (version 9.5.0). Further analysis using multivariate regression and PCA was done in R version 4.4.0. Codes for data analysis were publicly shared (https://github.com/herdiantrisufriyana/colab_ns1nst).

3. Results

3.1. Dengue patients' characteristics and clinical parameters

We included 86 patients with varying dengue severities, all of whom were 66 years old on average. Males comprised 56 percent of the sample, while females comprised 44 percent. According to dengue severity categories, Group A (DNWS) accounts for 25.5 % (n = 22), B (DWWS) 34.9 % (n = 30), and C (SD) with the most subjects in 39.5 % (n = 34) of all enrolled dengue patients. Seventy-two percent (n = 62) of dengue patients had primary infections, with the remainder having secondary infections (28 percent, n = 24). With a standard deviation of two days, the third-day post-fever was the typical sample collection time. The hospital stays of the patients ranged from 0 to 26 days, with an average of 12 days. The majority of patients (67 %) were found to have one or more comorbid conditions, the most prevalent of which were chronic renal failure (47 %), diabetes mellitus (31 %), and hypertension (16 %) (Table 1).

The average clinical parameters measured from the dengue patients showed white blood cell count, hemoglobin, and hematocrit results. Meanwhile, compared to the reference range, the platelet count was lower, the liver marker showed a huge concentration increase, and the renal function marker only showed a slight increase. This condition followed the dengue pathology, in which the platelet number was reduced and liver dysfunction was found (Table 1).

3.2. Febrile phase severity biomarker compared to the critical phase of DENV infection

There was no remarkable relationship between NS1 and NST to dengue severity during the first week of infection (Table S2). In the clinical settings, dengue progression phase was separated into the febrile phase and critical phase. To understand in phase-specific manner, we divided the dengue patient groups according to the day their samples were taken.

The samples collected between day zero and day three post-fever were classified as the febrile phase and the latter group as belonging to the critical stage of dengue infection. We compared NS1 and NST levels in the febrile (day 1–3 post fever onset) and critical (day 4–7 post fever onset) phases of acute dengue infection to investigate their levels in different dengue infection severities. Our findings revealed a substantial difference in NST levels between A to B and A to C during the febrile phase, not in the convalescent phase. All together indicates the importance of NST as an early marker for dengue severity (Fig. 1A, Table 2, and Table 3).

Other than NS1 and NST, we also observed other parameters that are generally measured in dengue patients. Only in the febrile stage, platelets were significantly lower in the more severe groups (Fig. 1B–Table 2). This result indicates good use of platelet prediction in the first three days of dengue infection. GPT was significantly increased

Table 1
Overall dengue patient demographic and clinical characteristics.

Indicators	Total Patient (n = 86)	Normal reference
Age (years old)	66 ± 16	–
Gender		
• Male (n)	48 (56 %)	–
• Female (n)	38 (44 %)	–
Severity		
• Dengue fever without warning signs (DNWS or A, n)	23 (27 %)	–
• Dengue fever with warning signs (DWWS or B, n)	29 (34 %)	–
• Severe dengue (SD or C, n)	34 (40 %)	–
Infection		
• Primary (n)	61 (71 %)	–
• Secondary (n)	25 (29 %)	–
Day of Fever (days)	3 ± 2	–
Hospital stays (days)	12 ± 14	–
Patients with comorbid (≧1, n)	58 (67 %)	–
Comorbid number (mean ± SD)	1 ± 1	–
Comorbid		
• Diabetes Mellitus (n)	27 (31 %)	–
• Hypertension (n)	14 (16 %)	–
• Chronic renal failure (n)	40 (47 %)	–
WBC (mean ± SD, 10^6/L)	6045 ± 3835	4000–10000
Hb (mean ± SD, gm/dL)	13.2 ± 2.4	♀ 12 - 15 ♂ 13 - 17
Hct (mean ± SD, %)	41.5 ± 15.9	♀ 36 - 47 ♂ 40 - 52
Platelet (mean ± SD, 10^9/L)	99 ± 85	150–400
GPT (mean ± SD, U/L)	127.8 ± 390.1	5–30
Cr (mean ± SD, mg/dL)	1.4 ± 1.3	0.8–1.3
NS1 (OD)	1.1 ± 1.0	–
NST (OD)	1.2 ± 0.9	–

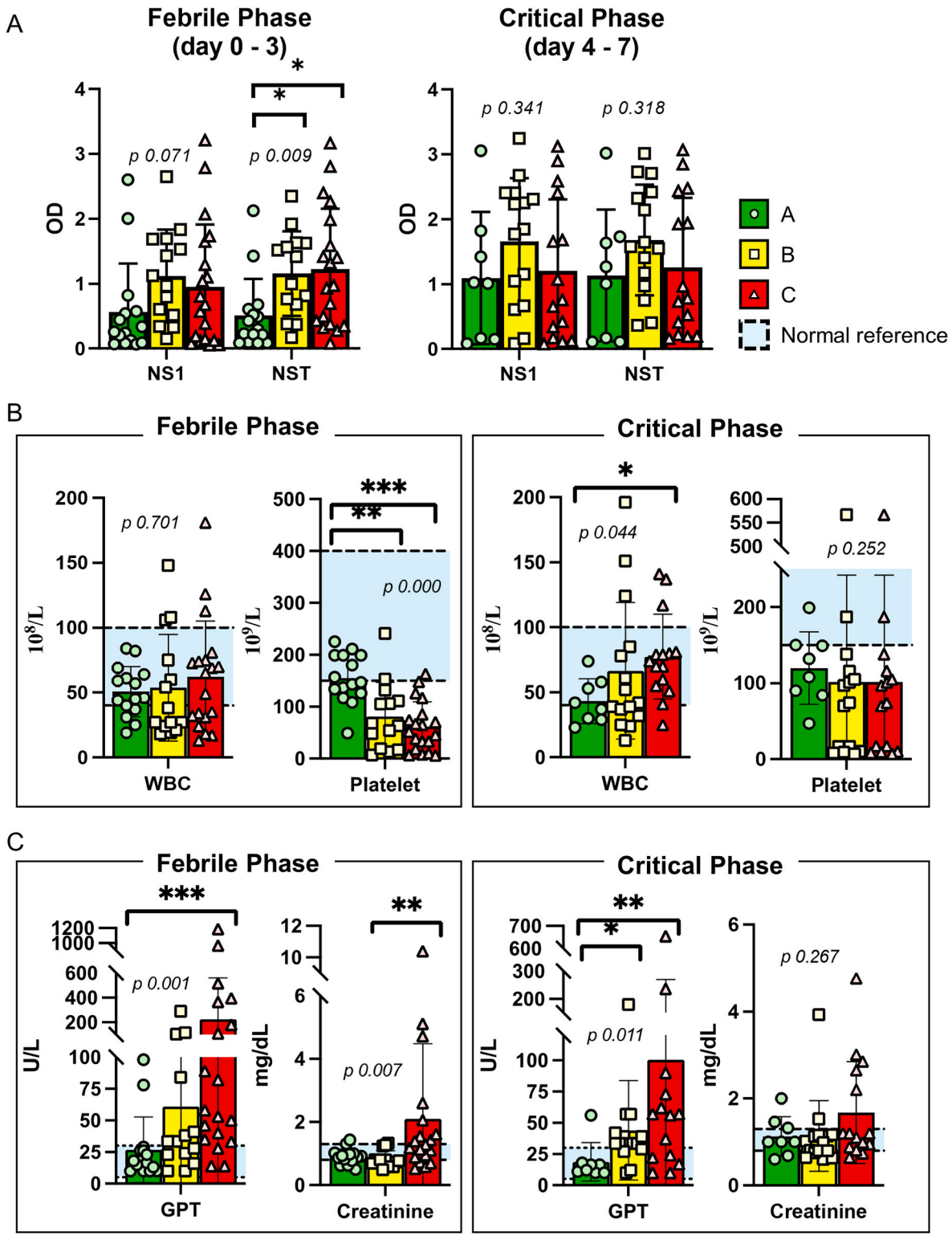

Fig. 1. NS1, NST, and several markers in the febrile and critical phases. (A) NS1 and NST concentrations in different febrile phases (Days 0–3) and critical phases (Days 4–7) of dengue severity infection. (B) Dengue patient white blood cell (WBC) and platelet numbers, with glutamic-pyruvic transaminase (GPT) and creatinine levels, respectively, in various severities in the febrile phase. (C) White blood cell and platelet numbers, GPT, and creatinine levels in the critical phase. Group A is dengue fever without warning signs, Group B is dengue fever with warning signs, and Group C is severe dengue or those treated in the ICU regardless of severity. Significance differences were tested using the independent-samples Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

in the group C as compared to group A in the febrile phase, and creatinine in B to C (Fig. 1C–Table 2). On the other hand, in the critical phase, we identified that GPT showed the most noticeable changes in mild dengue to the more severe groups of dengue infection (Fig. 1C–Table 3).

3.3. NS1 and NST exploration in various demographic and clinical backgrounds of acute dengue patients

The dengue patient age distribution was mostly older than 60

Table 2
Dengue patient febrile phase demographic, clinical and serologic marker concentrations in different dengue severities.

Indicators	A (n = 15)	B (n = 14)	C (n = 19)	*p-value*			
				Overall	A ↔ B	A ↔ C	B ↔ C
Day 0–3 post fever (Febrile Phase)							
Demographic							
Age (years old)	46 ± 18	72 ± 7	70 ± 12	**0.001**	**0.003**	**0.002**	1
Gender							
• Male (n)	11	5	12	**< 0.001**	-		
• Female (n)	4	9	7				
Clinical							
Infection							
• Primary (n)	12	12	12	**< 0.001**	-		
• Secondary (n)	3	2	7				
Day of Fever (days)	1.7 ± 1	2 ± 1	2.2 ± 0.9	0.382	-	-	–
Hospital stays (days)	0 ± 0	10.4 ± 6.5	18.5 ± 15.3	**< 0.001**	**< 0.001**	**< 0.001**	0.688
Comorbid number (mean ± SD)	0 ± 1	2 ± 1	2 ± 1	**0.001**	**0.003**	**0.007**	1
Comorbid							
• Diabetes Mellitus (n)	0	6	7	**< 0.001**	-		
• Hypertension (n)	2	7	12	**< 0.001**			
• Chronic renal failure (n)	1	2	4	**< 0.001**			
WBC (mean ± SD, 10^6/L)	5087 ± 1940	5371 ± 4103	6211 ± 4290	0.701	–	–	–
Hb (mean ± SD, gm/dL)	14.0 ± 1.4	13.0 ± 3.4	13.1 ± 2.4	0.248	–	–	–
Hct (mean ± SD, %)	42.1 ± 3.2	47.6 ± 37.4	40.5 ± 7.3	0.154	–	–	–
Platelet (mean ± SD, 10^9/L)	155 ± 47	81 ± 66	61 ± 48	**< 0.001**	**0.007**	**< 0.001**	1
GPT (mean ± SD, U/L)	26.7 ± 26.0	60.9 ± 74.4	224.4 ± 335.0	**0.001**	0.203	**< 0.001**	0.209
Cr (mean ± SD, mg/dL)	0.9 ± 0.2	0.8 ± 0.3	2.1 ± 2.4	**0.007**	0.904	0.135	**0.007**
Serological							
NS1 (OD)	0.6 ± 0.7	1.1 ± 0.7	1.0 ± 1.0	0.071	-	–	–
NST (OD)	0.5 ± 0.6	1.2 ± 0.7	1.2 ± 0.9	**0.009**	**0.019**	**0.026**	1

Table 3
Dengue patient critical phase demographic, clinical, and serologic marker concentrations in different dengue severities.

Indicators	A (n = 8)	B (n = 15)	C (n = 15)	*p-value*			
				Overall	A ↔ B	A ↔ C	B ↔ C
Day 4–7 post fever (Critical Phase)							
Demographic							
Age (mean ± SD, years old)	64 ± 18	70 ± 13	70 ± 12	0.849	-	-	–
Gender							
• Male (n)	3	6	11	**< 0.001**	-		
• Female (n)	5	9	4				
Clinical							
Infection							
• Primary (n)	7	10	9	**< 0.001**	-		
• Secondary (n)	2	5	6				
Day of Fever (mean ± SD, days)	4 ± 0	5 ± 1	5 ± 1	0.321	-	-	–
Hospital stay (mean ± SD, days)	0 ± 1	8 ± 4	25 ± 18	**< 0.001**	**0.005**	**< 0.001**	**0.010**
Comorbid number (mean ± SD)	1 ± 1	0	1 ± 0	0.192	-	-	–
Comorbid							
• Diabetes Mellitus (n)	2	5	7	0	–	–	–
• Hypertension (n)	4	3	12	0	–	–	–
• Chronic renal failure (n)	0	3	4	0	–	–	–
WBC (mean ± SD, 10^6/L)	4325 ± 1727	6653 ± 5244	7733 ± 3261	**0.044**	0.283	**0.016**	0.109
Hb (mean ± SD, gm/dL)	12.9 ± 1.8	12.8 ± 1.7	13.2 ± 2.9	0.711	–	–	–
Hct (mean ± SD, %)	39 ± 5.3	38.5 ± 5.5	40.5 ± 7.0	0.601	–	–	–
Platelet (mean ± SD, 10^9/L)	120 ± 48	92 ± 88	102 ± 140	0.252	–	–	–
GPT (mean ± SD, U/L)	19 ± 15	44 ± 40	311 ± 833	**0.011**	**0.030**	**0.003**	0.331
Cr (mean ± SD, mg/dL)	1.1 ± 0.5	1.1 ± 0.8	1.7 ± 1.2	0.267	–	–	–
Serological							
NS1 (mean ± SD, OD)	1.1 ± 1.0	1.7 ± 1.0	1.2 ± 1.1	0.341	–	–	–
NST (mean ± SD, OD)	1.1 ± 1.0	1.7 ± 0.9	1.3 ± 1.1	0.318	–	–	–

(Fig. 2A), showing a higher severe dengue prevalence in older patients than younger patients (Fig. S1). Older age also correlated with a slight increase in NS1 and NST concentrations (Fig. 2B). In order to confirm the concentration difference between ages, we separated the dengue patient population into adults (18–64 years old) and seniors (over or equal to 65 years old) and compared their NS1 and NST levels. The results showed no significant difference between NS1 and NST concentrations in either dengue severity group. Although some mean

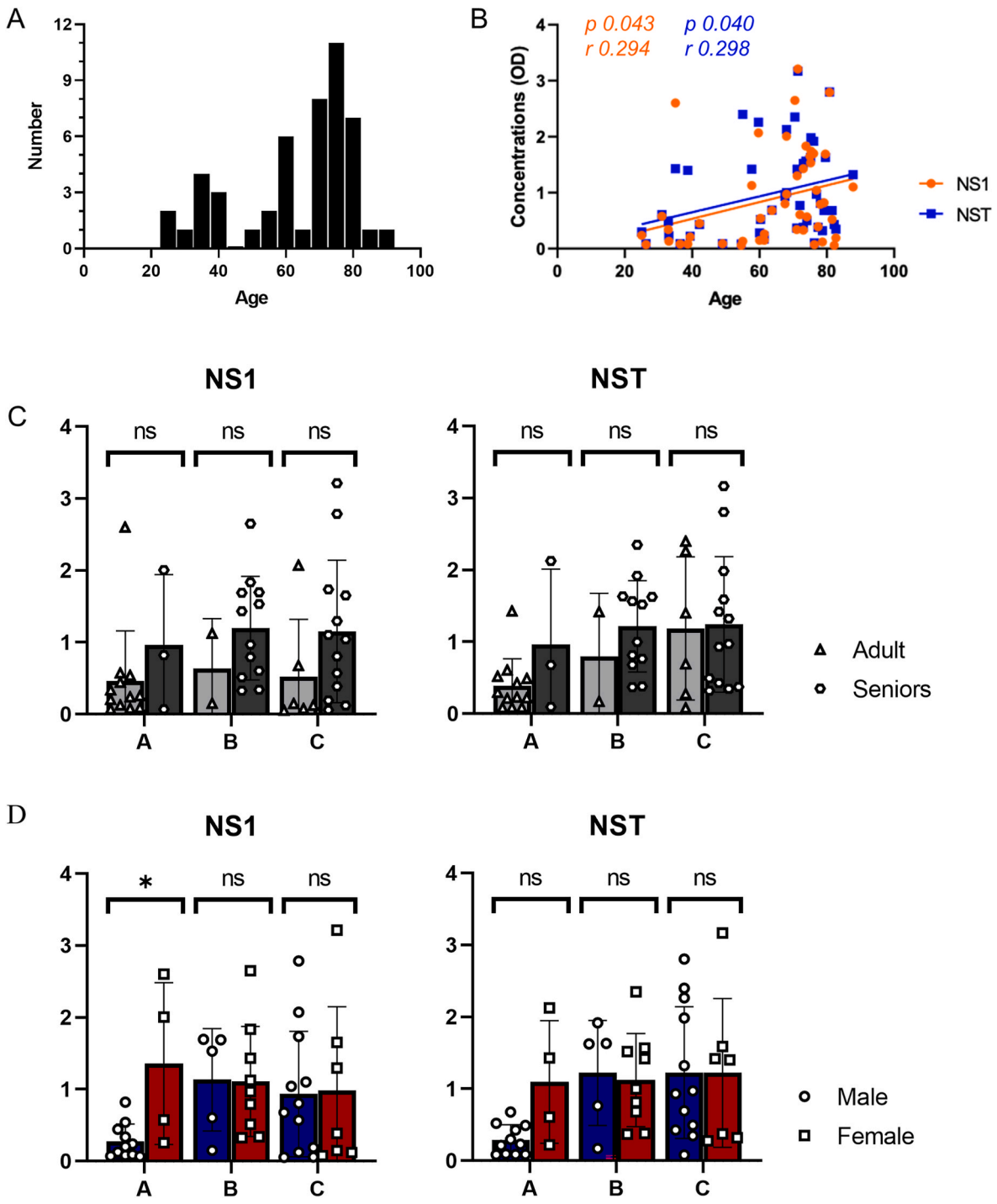

Fig. 2. NS1 and NST concentrations at various ages and sexes. (A) Dengue patient age distribution in the febrile phase of the disease. (B) Correlation test between age and NS1 and NST levels. (C) NS1 and NST concentrations in the febrile phase of adults (18–65 years old) and seniors (≥65 years old) dengue patients. (D) NS1 and NST concentrations in febrile phases in male and female dengue patients. Group A is dengue fever without warning signs, Group B is dengue fever with warning signs, and Group C is severe dengue or those treated in the ICU regardless of severity. Concentration was expressed as optical density (OD), and statistical analysis was performed using the independent-samples Mann–Whitney *U* test.

concentration differences in Groups A and B of NS1 and NST levels can be observed visually from the graph, in the C groups, the NST levels between adults and seniors are almost identical (Fig. 2C–Table S4). The findings suggest that NST has the potential to act as a severity marker in both adult and elderly populations, especially in severe dengue. Next, we compared NS1 and NST concentrations in male and female dengue patients. Our findings indicated that NS1 and NST levels were comparable in the B and C severity groups. However, in the A group, males exhibited significantly lower NS1 and NST levels than females (Fig. 2D–Table S4).

The potential impact of the onset day of fever on NS1 or NST concentrations was also examined. There was no discernible variation in dengue severity from Day 0 to day seven following the onset of fever from sample collection (Fig. 3A, Table S4). As a result, our findings suggest that the day of sample collection has no relevance to the amounts of NS1 and NST found in the blood. In patients with secondary

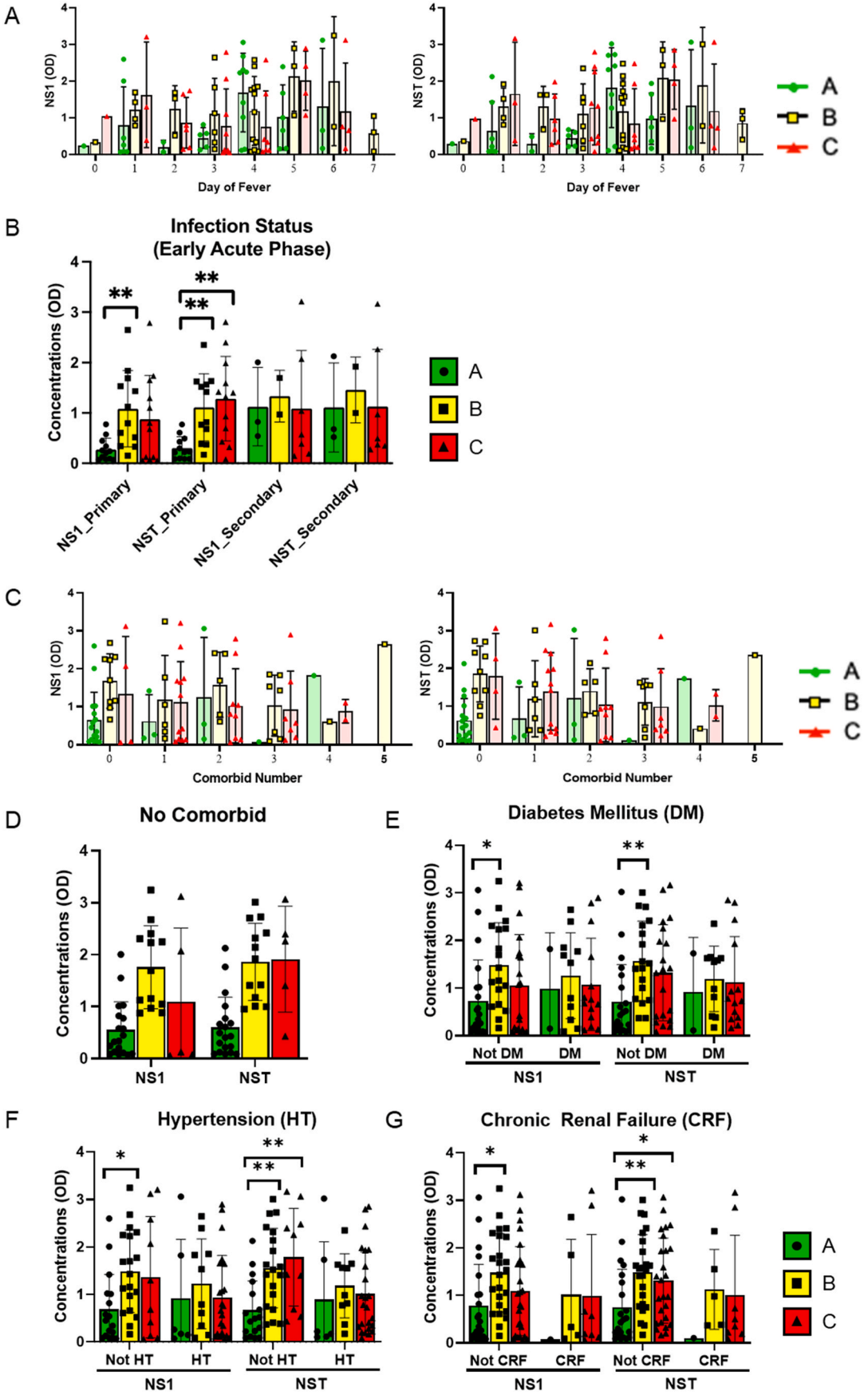

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Fig. 3. NS1 and NST concentrations on different days of fever (Days 0–7), infection status, and comorbid status. (A) NS1 and NST concentrations on each fever day in dengue patients. No marked difference was observed between the days of the samples obtained for various dengue severity patient conditions. (B) NS1 and NST concentrations in febrile phase dengue patients with primary and secondary infections. The primary infection showed a more significant difference between the DNWS to DWWS and SD, especially for NST, compared to those in the secondary infection in which levels between severity were not changed much. (C) NS1 and NST concentrations in the acute phase (days 1–3) of dengue patients showed no significant difference between patients with higher comorbid numbers and patients with lower comorbid numbers. NS1 and NST concentrations in febrile phase dengue patients with (D) no comorbid, (E) diabetes mellitus (DM), (F) hypertension (HT), and (G) chronic renal failure (CRF). A particularly exciting finding is that patients who do not have comorbidities, when compared to those with comorbidities, showed lower NS1 and NST concentrations in the milder form of the infection and higher concentrations in more severe infection. Group A is dengue fever without warning signs, Group B is dengue fever with warning signs, and Group C is severe dengue or those treated in the ICU regardless of severity. Statistical significance was tested using the Kruskal–Wallis test.

dengue infection, NS1 and NST levels were similarly expressed in the DNWS and SD groups, with DWWS showing the highest expression. The NS1 pattern in primary dengue infection mirrored that of the secondary infection, but DNWS and DWWS levels differed significantly. Unlike the DNWS values compared to DWWS and SD, the NST level in primary infection displayed a distinctive pattern with a modest increase in concentration. In contrast to the DNWS value to DWWS and SD, the NST level in the primary infection had a distinctive pattern with a modest concentration rise (Fig. 3B–Table S5).

We examined the correlation between the number of comorbidities in dengue patients and their NS1 and NST levels. Our analysis did not reveal any significant relationship (Fig. 3C), despite the known increased risk of morbidity and fatality associated with comorbidities in dengue patients.[27,28] Further analysis of the relationship between the number of comorbidities in dengue patients and their NS1 and NST levels did not reveal any significant association (Fig. 3D). Additionally, we measured NS1 and NST levels in the top three comorbidities found in our dengue patient samples, DM, HT, and CRF. Those with comorbidities do not show significant differences between NS1 and NST in dengue severity. Meanwhile, in those without any comorbidities, all NS1 and NST in the DWWS were significantly higher than those in the DNWS. Hypertension and CRF also showed a more increased NST in SD to the DNWS (Fig. 3E, F, 3G).

3.4. The relationship between NS1, NST, and other variables

Understanding the complexity of dengue disease, we analyze the relationship of NS1 and NST to other clinical markers using multivariate regression analysis. Using the ABC severity grouping as the outcome, NS1 and NST were measured and compared with other variables. However, it's still unknown whether NST influences severity or the other way around. We tested both directions to prove our hypothesis.

First, we test the assumption of severity influencing the NST levels. Our results showed the effect of sex (OR = 0.655 (0.456–0.940), adjusted p-value 0.024), age (OR = 1.476 (1.023–2.128), adjusted p-value 0.04) and APTT (OR = 0.000 (0.000–0.397), adjusted p-value 0.049) on severity. Meanwhile, the effect of NS1 (OR = 2.506 (2.333–2.692), adjusted p-value <0.001) on severity with age as covariates (Table 4, Table 5, Fig. S2). The effects of NS1 were further adjusted using its confounders (age) and mediators (outcome) (OR = 2.517 (2.336–2.711), adjusted p-value <0.001). Since the sample size only allowed for one covariate in addition to NS1, we reduced the number of covariates without leaking information about the dependent variable as the outcome using PCA (Fig. S4). There was no significant effect of age and sex on the NST beyond the severity effect. In contrast, the effect of NS1 on the NST persisted without the influence of severity. There was no mediator found between APTT and the NST (**Table 6**).

Second, the assumption of NST levels influencing severity as outcomes was tested. Our results showed the effect NS1 (OR = 2.467 (1.171–5.197), adjusted p-value = 0.018) on severity after adjustment for age as the confounder (Table 7, Table 8, Fig. S3). The effects of NS1 were further adjusted using its confounders (age) and mediators (albumin and NST). Similar to the above, we used PCA for further analysis due to sample limitation (Fig. S5). The analysis showed no significant effect of NS1 on the outcome beyond the NST effect (p 0.108, OR = 0.085 (0.004–1.719)). In contrast, the effect of NS1 on the severity persisted without any influence of albumin (**Table 9**).

3.5. Platelet count and NST are essential biomarkers in predicting dengue progression

We included platelets in the prediction analysis with NS1 and NST since it is a simple clinical characteristic to collect but is identifiable in different severities (Fig. 1B). In 3 comparison groups (in this part, severity grouping of A, B, and C was used instead of DNWS, DWWS, and SD to reduce the writing length; A vs. BC, A vs. B, and A vs. C), platelets and NST were effective predictors, while NS1 was only in 2 comparison groups (A vs. BC and A vs. B). Further analysis revealed that in all group comparisons for dengue, platelets or NST displayed a greater AUC area than NS1 (**Table 10**). This result indicates the promising capability of NST as a dengue severity biomarker more superior to NS1.

4. Discussion

This is the first study to compare the NST complex to NSI in terms of its potential to predict severe dengue and also its relationship to other variables. Our analysis of 86 dengue patients revealed that despite no correlation between NS1 and NST and the severity of the infection through the first week, NST levels significantly differed between the mild dengue to moderate and severe groups during the first three days of fever onset. In contrast, NS1 revealed fewer pronounced differences in severity between these groups. According to previous findings, the first three days following the onset of the fever were when the highest percentage of NS1 ELISA positivity (92 percent) was recorded.[29] Accordingly, rapid measurement of NS1 antigen was widely used as an early diagnosis of DENV infection in the febrile phase, as its expression is highly associated with viral replication.[30,31] Indeed, the levels of NST were elevated following the presence of soluble NS1 since the first day of fever. It is proposed that NST can be used for assessing dengue infection and evaluating dengue severity.

Table 4
Univariate regression analysis of the first assumption.

Variable	Term	Estimate	Std.error	Statistic	p-value	OR	LB	UB
NS1 *	value	0.907	0.035	25.767	**<0.001**	2.476	2.311	2.653
Outcome*	B	0.894	0.221	4.051	**<0.001**	2.444	1.586	3.766
Outcome*	C	0.626	0.218	2.878	**0.005**	1.871	1.221	2.866
Sex*	M	−0.424	0.184	−2.298	**0.024**	0.655	0.456	0.940
Age*	>65	0.389	0.187	2.084	**0.04**	1.476	1.023	2.128
APTT*	value	−50.557	25.324	−1.996	**0.049**	0.000	0.000	0.397

Table 5
Multivariate regression analysis of the first assumption. The analysis was done to adjust the effects of NS1 using its confounders. Severity is noted as the outcome.

Variable	Covariates	Term	Estimate	Std Error	Statistic	p-value	OR	LB	UB
NS1*	Age	OD value	0.919	0.036	25.180	**<0.001**	2.506	2.333	2.692
Sex*		M	−0.424	0.184	−2.298	**0.024**	0.655	0.456	0.940
Age*		>65	0.389	0.187	2.084	**0.04**	1.476	1.023	2.128
APTT*		value	−50.557	25.324	−1.996	**0.049**	0.000	0.000	0.397
Outcome	Age + NS1 + sex	B group	0.130	0.091	1.423	0.158	1.139	0.952	1.36

Note: * p-value≤0.05.

Table 6
Mediation analysis of the first assumption. The mediation analysis confirmed whether the NS1 effect on the dependent variable persisted without the mediator effect, including NST. A non-significant result means the effect persisted only through the mediators.

Variable	Covariates	Mediators	Term	Estimate	Std Error	Statistic	p-value	OR	LB	UB
NS1 *	Age	Outcome	value	0.923	0.038	24.281	**<0.001**	2.517	2.336	2.711
Sex		Outcome	M	−0.292	0.184	−1.584	0.117	0.747	0.520	1.072
Age		NS1 + outcome	>65	−0.092	0.071	−1.298	0.198	0.912	0.794	1.048
Age		NS1	>65	−0.083	0.069	−1.213	0.228	0.920	0.805	1.053
Age		Outcome	>65	0.147	0.192	0.764	0.447	1.158	0.795	1.687

Table 7
Univariate regression analysis of the first assumption.

y.level	variable	term	estimate	std.error	statistic	p.value	OR	LB	UB
BC vs. A	Age*	>65	1.831	0.517	3.543	<0.001	6.243	2.267	1.719500e+01
BC vs. A	NST *	value	1.296	0.392	3.303	0.001	3.656	1.694	7.888000e+00
BC vs. A	Viral load*	positive	1.692	0.532	3.178	0.001	5.429	1.912	1.541100e+01
BC vs. A	Platelet*	value	−0.013	0.004	−3.354	0.001	0.987	0.980	9.950000e-01
BC vs. A	Albumin*	value	−1.322	0.431	−3.071	0.002	0.266	0.115	6.200000e-01
BC vs. A	NS1*	value	1.068	0.361	2.959	0.003	2.910	1.434	5.905000e+00
BC vs. A	Hipertension*	yes	1.556	0.555	2.802	0.005	4.742	1.597	1.408300e+01
BC vs. A	DM*	yes	2.054	0.778	2.641	0.008	7.800	1.698	3.583000e+01
BC vs. A	GOT*	value	0.008	0.003	2.215	0.027	1.008	1.001	1.014000e+00
C vs. B	Sex*	M	1.526	0.529	2.885	0.004	4.600	1.631	1.297300e+01
C vs. B	Hipertension *	yes	1.522	0.529	2.875	0.004	4.582	1.624	1.293000e+01
C vs. B	Platelet*	value	−0.008	0.004	−2.131	0.033	0.992	0.984	9.990000e-01
C vs. B	Creatinin*	value	1.342	0.682	1.966	0.049	3.826	1.004	1.457400e+01

Table 8
Multivariate regression analysis of the second assumption. The analysis was done to adjust the effects of NS1 using its confounders.

Variable	Covariates	Term	Estimate	Std.Error	Statistic	p-value	OR	LB	UB
NS1*	age	Value	0.903	0.38	2.375	0.018	2.467	1.171	5.197

Note: * p-value≤0.05.

Table 9
Mediation analysis of the second assumption. The mediation analysis was done to confirm whether the NS1 effect on the dependent variable persisted without the mediator effect, including NST.

Variable	Covariates	Mediators	Term	Estimate	Std.Error	Statistic	p-value	OR	LB	UB
NS1*	age	Albumin	Value	0.919	0.376	2.442	0.015	2.508	1.199	5.245
NS1	age	NST	Value	−2.461	1.532	−1.606	0.108	0.085	0.004	1.719
NS1	age	Albumin + NST	Value	−0.249	0.537	−0.463	0.643	0.780	0.272	2.236

Table 10
NS1 and NST prediction ability in various groups of severe dengue. The results are presented as the AUC analysis results. Group A is dengue fever without warning signs, Group B is dengue fever with warning signs, and Group C is severe dengue or those treated in the ICU regardless of severity. *p value < 0.05, **p value < 0.01.

Acute Phase (Day 1–3)	A to BC	A to B	A to C	B to C
Platelet	0.873***	0.838***	0.898***	0.581
NS1	0.677*	0.771**	0.607	0.391
NST	0.778***	0.81***	0.754*	0.477

The levels of NS1 and NST were also compared concerning patient characteristics such as age, sex, day of fever, infection status, comorbidities, and correlation with dengue patient blood parameters. Our findings indicated that senior citizens tended to have higher levels of NS1 and NST, but that NS1 differences between adults and seniors were more prevalent than the NST differences in either severity category. Although NS1 and NST levels were unaffected by sex, we discovered a significant sex discrepancy in the groups of people with mild dengue. This gap may be caused by the sample's small number of female patients (n = four). In addition, the day of the fever did not affect the levels of NS1 or NST, indicating that the day the sample was obtained would not

impact the patient's decision-making. On the other hand, secondary infection of the dengue virus demonstrated a propensity for increased NS1 or NST concentrations across all severity categories. Compared to more severe types, mild illnesses have a lower concentration in the initial infection. However, the sensitivity of NS1 detection in secondary infections requires further validation. At the same time, different observations were reported, which probably depended on the presence of IgG and/or IgM immunocomplexes,[18,30,32,33] and other possible interactions with host factors based on our current study.[19]

Comparable patterns were found in patients without comorbidity conditions and those with diabetes, hypertension, and chronic renal failure. In the meantime, the levels of NS1 and NST were unaffected by a patient's comorbidity. In contrast, comorbidity conditions displayed a high association with severe dengue progression and fatality[34,35]; the levels of NS1 and NST in the patients classified as high-risk needed more validation.

In this study, we are trying to understand the role of NST in severe dengue. The question of whether severity causes NST increase or the other way was tested here. We tried two assumptions in this test, the first one is severity influences NST levels, and the second, severity is influenced by NST. Our results showed although older age (over 65 years old) influenced NS1 level, its influence on severity needs to be done through NST. Another report in 2013 proposed the role of NS1 in inducing Anti-Thrombin Antibodies (ATAs) in dengue patients that might bind to plasminogen further interfering with the coagulation system.[36]

As our analysis used soluble NS1 (sNS1) in the blood, we should understand the factors influencing its release. Secreted DENV NS1 is mainly formed as a complex with high-density lipoprotein (HDL).[37,38] Several factors influencing dengue NS1 production and secretion were virus serotypes, a mutation in the NS1 gene, exocytosis-related complexes, and exocysts. Between serotypes 1 to 3, DENV1 was reported to have the highest NS1 secretion; DENV3 had the lowest NS1 secretion. In the NS1 gene mutations, the change of Trp68 → stop codon and Val236 → Ala accounted for the suppression of NS1 production and release in the DENV3 serotypes.[39] In exocytosis, an octameric protein complex called the exocyst was thought to be responsible for anchoring secretory vesicles to the plasma membrane before SNARE-mediated fusion.[40] One of its proteins was named exocyst complex component 7 (EXOC7, formerly known as Exo70). In the dengue-infected patient, Exo70 levels increased from 18 h post-infection. Meanwhile, the knockdown of Exo70 resulted in a lower virus titer by inhibiting DENV secretion from the infected cells.[41]

Dr. Paul Young from Australia using E. M. found that the dengue virus replication complex utilizes NS1 in its process.[12,30] Given that NS1 is associated with viral replication, it is reasonable to suppose that the release of DENV from infected cells is likewise correlated with NS1 secretion, which might also be affected by Exo70 protein levels. Based on these findings, these possible complexes and the potential events for regulating DENV and NS1 protein release, leading to dynamic changes in NST complex protein expression, should be considered. To summarize, Exo70 expression level may influence NS1 secretion and as a result, NST level, which in turn determines the disease progression in dengue infection.

The limitation of this study was caused by the small sample size, which made the analysis interpretation more challenging due to patient heterogeneity. The imbalance in sampling between males and females in each severity grouping may also pose some influence on the precision of the results. In addition, further studies investigating what triggers NS1 secretion are still needed to gain a greater understanding. Confirmation studies on NS1 and NST concentrations as dengue severity biomarkers in larger dengue patient settings are also required to comprehend their role in more detailed patient conditions, such as comorbidity.

CRediT authorship contribution statement

Josephine Diony Nanda: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Trai-Ming Yeh:** Resources, Supervision, Writing – review & editing. **Rahmat Dani Satria:** Conceptualization, Formal analysis, Methodology. **Ming-Kai Jhan:** Formal analysis, Investigation, Methodology. **Yung-Ting Wang:** Formal analysis, Investigation, Methodology. **Ya-Lan Lin:** Investigation, Methodology. **Herdiantri Sufriyana:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. **Emily Chia-Yu Su:** Investigation, Software, Supervision. **Chiou-Feng Lin:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Tzong-Shiann Ho:** Conceptualization, Investigation, Resources, Supervision, Writing – review & editing.

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Appendix A. Supplementary data

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